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Association of Cysteine-Rich Secretory Protein 3 and β-Microseminoprotein with Outcome after Radical Prostatectomy

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Abstract

Purpose—It has been suggested that cysteine-rich secretory protein 3 (CRISP-3) and β-microseminoprotein (MSP) are associated with outcome in prostate cancer. We investigated whether these markers are related to biochemical recurrence and whether addition of the markers improves prediction of recurring disease.

Experimental Design—Tissue microarrays of radical prostatectomy specimens were analyzed for CRISP-3 and MSP by immunohistochemistry. Associations between marker positivity and postprostatectomy biochemical recurrence [prostate-specific antigen (PSA) >0.2 ng/mL with a confirmatory level] were evaluated by univariate and multivariable Cox proportional hazards regression. Multivariable analyses controlled for preoperative PSA and pathologic stage and grade.

Results—Among 945 patients, 224 had recurrence. Median follow-up for survivors was 6.0 years. Patients positive for CRISP-3 had smaller recurrence-free probabilities, whereas MSP-positive patients had larger recurrence-free probabilities. On univariate analysis, the hazard ratio for patients positive versus negative for CRISP-3 was1.53 ($P = 0.010$) and for MSP was 0.63 ($P = 0.004$). On multivariable analysis, both CRISP-3 ($P = 0.007$) and MSP ($P = 0.002$) were associated with recurrence. The hazard ratio among CRISP-3– positive/MSP-negative patients compared with CRISP-3– negative/MSP-positive patients was 2.38. Adding CRISP-3 to a base model that included PSA and pathologic stage and grade did not enhance the prediction of recurrence, but adding MSP increased the concordance index minimally from 0.778 to 0.781.

Conclusion—We report evidence that CRISP-3 and MSP are independent predictors of recurrence after radical prostatectomy for localized prostate cancer. However, addition of the markers does not importantly improve the performance of existing predictive models. Further research should aim to elucidate the functions of CRISP-3 and MSP in prostate cancer cells.

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Prostate cancer is one of the most frequent male cancers in Western countries (1). Patient outcomes after therapy for prostate cancer can be predicted from individual clinical features (serum prostate-specific antigen [PSA], digital rectal examination) or pathologic features (Gleason score, extent of disease). Combining these factors, however, increases predictive accuracy, and a number of models that combine clinical and/or pathologic factors have been developed to predict an individual patient's probability of disease recurrence or survival after treatment of prostate cancer (2–6). The postoperative nomogram originally developed by Kattan et al. (2) is widely used by clinicians to predict freedom from disease recurrence for patients who have undergone radical prostatectomy (RP), and it was recently updated to predict the 10-year probability adjusted for the disease-free interval after RP (7). Despite the widespread use of the postoperative nomogram and its reasonable accuracy, better tools are needed to predict an individual patient's probability of disease recurrence after RP.

Many reports have described tissue markers of prognostic value in prostatic tumors, but attempts to use such biomarkers to improve the predictive accuracy of existing nomograms have been largely unsuccessful. Limiting factors include the lack of optimized, standardized procedures for processing of tissue from RP specimens, although efforts to optimize tissue fixation have been described (8). Another limiting factor is the lack of methods to reliably quantify immunohistochemical staining.

Cysteine-rich secretory protein 3 (CRISP-3), also known as specific granule protein of 28 kDa (SGP28), was first discovered in human neutrophils, and its cDNA was cloned from a human bone marrow cDNA library (9). In humans, CRISP-3 mRNA has been detected at high concentrations in salivary glands, pancreas, and prostate (10), and CRISP-3 protein has been detected in human body fluids, including saliva, sweat, blood, and seminal plasma (11). We have previously shown that CRISP-3 is widely distributed in the secretory epithelium of the male reproductive tract with particularly intense expression in the epididymis and the ampullary part of the deferent ducts (12). The function of CRISP-3 in humans remains to be established, although a role in innate immune defense has been hypothesized. This hypothesis is supported by the high expression level in neutrophils and in exocrine glands (11) and by sequence similarities with so-called pathogenesis-related proteins, which are involved in plant antimicrobial defense (13). Further supporting a role in the immune response, CRISP-3 seems to be overexpressed in chronic pancreatitis (14). In human neutrophils, CRISP-3 is localized in specific and gelatinase granules, which are partially exocytosed during neutrophil migration (15).

Two independent groups have reported CRISP-3 mRNA to be expressed at low levels in benign prostate tissues but highly overexpressed in prostate cancer (16–18). Ernst et al. showed a 20-fold increase in CRISP-3 mRNA in prostate cancer using microdissection of malignant and benign prostate tissues followed by reverse transcription-PCR (RT-PCR; ref. 18). Asmann et al. did electronic profiling of expressed sequence tags to identify differentially expressed genes in normal and malignant prostate, and among the 600 genes profiled, the most highly up-regulated was CRISP-3 (16). By RT-PCR of laser capture microdissected tissues, they showed CRISP-3 expression to be increased by a factor of 50 to 300 in malignant versus benign prostate tissues; CRISP-3 was therefore proposed as a new biomarker for prostate cancer (16,17). We recently showed overexpression of CRISP-3 in primary prostate tumors and metastases by immunohistochemistry (IHC) and *in situ* hybridization (19).

Using immunoprecipitation and gel filtration of seminal plasma proteins combined with the examination of the isolated proteins, we found that CRISP-3 forms very high-affinity noncovalent complexes with β-microseminoprotein (MSP; ref. 20), one of the most abundant proteins secreted from the prostate gland (21). MSP is also known as prostate secretory protein of 94 amino acids (PSP94), β-inhibin, prostatic inhibin peptide, and immunoglobulin binding

factor. It is expressed in the benign and malignant prostatic epithelium (22–26). Immunohistochemical and *in situ* hybridization studies have suggested that MSP is an independent prognostic factor for survival in prostate cancer patients (27–29). The functions of MSP in humans remain to be elucidated.

The aim of the present study was to investigate whether the expression of CRISP-3 and MSP in prostate tissue can predict disease recurrence after RP and whether the performance of a posttreatment-predictive nomogram can be improved by the addition of these biomarkers.

Materials and Methods

Patients

Approval from the institutional review board of Memorial Sloan-Kettering Cancer Center (MSKCC) was obtained before initiating the study, and the Helsinki Declaration regarding the use of human tissues was strictly observed. The study included consecutive patients treated with RP for localized and locally advanced prostate cancer at MSKCC between 1985 and 2003. Presurgical and follow-up information was compiled on all patients from our prospective database. We excluded patients who received treatments (such as radiation or androgen deprivation therapy) as neoadjuvant to or immediately after prostatectomy. Initially, 1,073 consecutive patients who fulfilled our criteria were selected. Subsequently, we also excluded all cases that did not have pathologic material (original H&E slides or paraffin blocks) available for our review, leaving 947 patients who constituted the subject of this study. Two patients were not followed for biochemical recurrence (BCR), leaving 945 patients in the cohort for analysis. Descriptive characteristics of the patient cohort are given in Table 1. BCR was defined as PSA >0.2 ng/mL confirmed by a subsequent higher value (30).

Creation of tissue microarrays

H&E slides of the prostatectomy specimens were reviewed by two of the authors (H.A.A., V.E.R.) blinded to clinical outcome. Areas with tumor were marked, and corresponding paraffin blocks were retrieved. For each patient, at least one block representative of the tumor was selected, and tissue cores of 0.6 mm were punched out in triplicate from locations randomly selected within the marked tumor areas. Tissue cores were mounted in a blank recipient block using either a manual or automated tissue microarrayer (Beecher Instruments Inc.), depending on their availability. The resulting 13 multiblock tissue microarray blocks were made ready for future sectioning.

Antibodies

Polyclonal rabbit anti–CRISP-3 antibodies were raised against human CRISP-3 protein purified from neutrophil granulocytes. Isolated neutrophils were stimulated for exocytosis with phorbol-12-myristate-13-acetate in the presence of protease inhibitors. CRISP-3 was isolated from the exocytosed material by affinity with α-1-B-glycoprotein (A1BG) and further purified by cation-exchange chromatography as previously described (31). The immunostaining pattern in benign and malignant prostate tissue specimens was identical to that of a polyclonal anti–CRISP-3 immunoglobulin G (IgG) preparation raised against a recombinant fusion protein that we have used in previous studies (data not shown; refs. 11,12,19). In addition, by Western blot of tissue homogenates of benign prostatic hyperplasia and prostate cancer, both antisera identified identical bands of 29 and 27 kDa, corresponding to N-glycosylated and nonglycosylated CRISP-3; the antisera identified no additional protein bands (data not shown). Immunostaining of MSP was done with a previously described polyclonal affinity-purified anti-MSP IgG preparation (code P4; ref. 23).

Immunohistochemistry

Immunohistochemical staining of CRISP-3 and MSP was done using EnVision Detection System Peroxidase/DAB, Rabbit/Mouse (code K5007, DAKO) and DAKO TechMate 500/1000 staining machine (BioTek solutions). Briefly, 4-μm sections were mounted on Superfrost plus slides (Fisherbrand, Fisher Scientific International Inc.), deparaffinized, rehydrated, and incubated with Target Retrieval Solution (pH, 9.9; DAKO) and sequentially heated in a microwave oven at 900, 750, 650, and 300 W for 2 min at each energy level. The sections were incubated with polyclonal anti–CRISP-3 and anti-MSP IgGs at a final dilution of 3.5 and 0.3 μg/mL and further processed using the EnVision Detection System Peroxidase/DAB, Rabbit/Mouse with biotinylated secondary antibodies, goat anti-rabbit, and goat anti-mouse IgGs, (DAKO A/S, Glostrup, Denmark). Finally, the sections were counterstained with Mayer's hematoxylin solution, and coverslips were applied with Pertex mounting medium (Histolab Products AB). Negative control experiments, including processing of slides with nonimmune rabbit IgG and specific preabsorption of the antibodies by adding excess amount of antigen, were described earlier (12,23).

The immunohistochemical stains were evaluated by two of the authors (A.S.B., H.A.A.). Only cytoplasmic staining was considered positive. The expression was assessed semiquantitatively for both the extent and intensity of tumor immunoreactivity. Each individual tissue core was reported independent of the results of the remaining two cores from the same patient. The percentage of positive tumor cells was estimated and assigned values of 0, 5, or multiples of 10% to 100%. The intensity of the expression was assigned a value of 0, 1, 2, or 3. When high-grade prostatic intraepithelial neoplasia (HGPIN) or non-neoplastic prostatic tissue was present, staining results of these tissues were evaluated separately.

Statistical analysis

Immunostaining intensity and percentage of positive tumor cells was defined for each patient to be the mean of the values of the three cores. The presence of tumorous tissue in at least two interpretable cores was required to include a case in the statistical analysis; patients with ≤1 core scored were treated as missing for that marker. For CRISP-3, 139 patients had ≤1 sample scored for both percentage of positive tumor cells and immunostaining intensity. For MSP, 166 patients had ≤1 sample scored for immunostaining intensity and an additional patient had ≤1 sample scored for percentage of positive tumor cells.

We used univariate Cox proportional hazards regression to evaluate the association between marker positivity and BCR (PSA >0.2 ng/mL with a confirmatory level) following prostatectomy. Because prior data on CRISP-3 and MSP were limited, we tested numerous different cutoffs for defining a patient as marker positive. Given the consequent multiple testing, we were aware that our results could only be hypothesis generating, and that any significant associations would have to be further tested in an independent data set. However, we did not formally adjust *P* values for multiple testing. For the percentage of positive tumor cells, we varied the cut-point for a positive result from 10% to 90% in steps of 10%; for immunostaining intensity, we varied the cut-point from 0.5 to 2.5 in steps of 0.5. We also tested cut-points that combined the percentage of positive tumor cells and immunostaining intensity. Recurrence-free probabilities were obtained using Kaplan-Meier methodology and were compared by the log-rank test.

Predictive accuracy was defined in terms of the concordance index (*c* index). In brief, the *c* index is comparable to the area under the receiver-operating characteristic curve and can be used to quantify discrimination for survival time data in single-variable and multivariable models. All *c* indices were bootstrap corrected with 200 replications. Statistical analyses were conducted using Stata 9.0 (StataCorp LP).

Results

Immunohistochemical evaluation of CRISP-3

The cytoplasmic expression of CRISP-3 in tumor tissue varied greatly among tumors but was extensive (>50% of tumor cells staining) in the majority of cases (447 patients or 55%; Fig. 1A). The intensity of staining was also variable, as 597 cases (74%) showed staining intensity of 1 and 237 cases (29%) showed intensity of 2 or stronger. When present, CRISP-3 expression tended to be uniform across the tissue, with minimal variability in its intensity. In some cases, however, there was a stronger apical condensation toward the glandular lumen, as expected for a secretory protein. More details about the extent and intensity of CRISP-3 expression are available in Table 2. We identified HGPIN in 76 patients. CRISP-3 expression within HGPIN was present in 71 patients (93%) and was strong and extensive in 46 patients (Fig. 1E). Most benign epithelial cells lacked CRISP-3 expression or stained weakly. Occasionally, in the stromal compartment, we found neutrophilic infiltrate with strong immunoreactivity, confirming previous observations of CRISP-3 expression in neutrophils.

The distribution of CRISP-3 expression is shown in Fig. 2A and B. Among the 806 patients with complete CRISP-3 data, the majority had high expression of CRISP-3, with 597 (74%) having immunostaining intensity ≥1 and 567 (70%) having ≥30% of tumor cells positive.

Immunohistochemical evaluation of MSP

Because MSP is one of the three predominant secreted proteins of the prostate gland, benign epithelium adjacent to tumor was strongly immunoreactive in all cores in which it was present (Fig. 1D). In most cancers, only a small fraction of cells showed MSP expression, with 386 (50%) showing <10% of tumor cells immunoreacting and only 142 cases (18%) showing >50% of tumor cells immunoreacting. In addition, overall staining intensity was weak. In most cases, MSP immunoreactive tumor cells were often scattered and of varying intensity, contrasting the more uniform intensity of CRISP-3 staining. Rarely, however, we found tumors with homogeneous strong immunoreactivity.

HGPIN was positive for MSP in all 77 cases in which it was found (Fig. 1F). In 74 of these cases, staining intensity was ≥1, and >20% of the cells in HGPIN areas were stained.

The distribution of MSP expression is shown in Fig. 2C and D. Overall, there were 778 patients with complete MSP data. In contrast to CRISP-3, most patients had low expression of MSP. Only 22% (170/779) had immunostaining intensity ≥0.5, and only 50% (386/778) had ≥10% of positive tumor cells.

Prediction of recurrence

Among all 945 patients, there were 224 recurrences. Median follow-up for survivors was 6.0 years. The results of univariate analyses are shown in Table 2. For both CRISP-3 and MSP, immunostaining intensity alone was not predictive of BCR. Percentage of positive tumor cells, however, seemed to be associated with recurrence when cut-points were 50% to 90% for CRISP-3 and 10% to 40% for MSP. We also tested cut-points that combined staining intensity and percentage of positive cells. For CRISP-3, we identified a distinct subpopulation of tumors with strong immunostaining and with a majority of the tumor cells positive. Based on this finding, we tested cut-points that defined CRISP-3 positivity as mean immunostaining intensity of 1.5 or more combined with ≥70% or ≥80% of tumor cells positive. For MSP, based on previous publications and on the hypothesis that even a small number of MSP-expressing tumor cells may indicate poor outcome, we tested cut-points of staining intensity of 1 or more combined with ≥20% or ≥30% of tumor cells immunoreactive. All four of these cut-points yielded significant association with recurrence in univariate analysis (Table 2). With the

CRISP-3 cut-point of intensity ≥1.5 and ≥80% of tumor cells positive, the hazard ratio for patients positive versus negative for CRISP-3 was 1.53 [95% confidence interval (95% CI), 1.11, 2.12; $P = 0.010$]. Patients with CRISP-3–positive tumors by this definition also had smaller recurrence-free probabilities (Fig. 3A). With the MSP cut-point of intensity ≥1.0 and ≥20% of tumor cells positive, the hazard ratio for patients positive versus negative for MSP was 0.63 (95% CI, 0.46, 0.86; $P = 0.004$), and MSP-positive patients had larger recurrence-free probabilities (Fig. 3B). For the remaining analyses, we classified patients as CRISP-3–positive and MSP-positive based on these cut-points.

To determine if CRISP-3 or MSP could aid in clinical decision making, we compared a base model incorporating well-established predictors of biochemical failure (preoperative PSA, pathologic Gleason score, and presence or absence of extracapsular extension, seminal vesicle invasion, positive surgical margins, and lymph node involvement) to a model combining the base model variables and marker status. On multivariable analysis, CRISP-3 was significantly associated with recurrence (HR, 1.59 for positive versus negative; 95% CI, 1.13, 2.22; $P = 0.007$). The *c* index of the base model among patients with complete CRISP-3 data was 0.780. Adding CRISP-3 status to this model did not enhance predictive accuracy (*c* index, 0.777). On multivariable analysis, negative MSP status was significantly associated with recurrence (HR, 0.59 for positive versus negative; 95% CI, 0.42, 0.83; $P = 0.002$). Among patients with complete MSP data, the *c* index of the base model was 0.778. Adding MSP status marginally increased the *c* index to 0.781.

We hypothesized that patients with a high expression of CRISP-3 would have a low expression of MSP. There were 743 patients with complete data for both CRISP-3 and MSP. Among 138 patients positive for CRISP-3, 97 (70%) were negative for MSP. However, among the 605 patients negative for CRISP-3, the percentage negative for MSP was similar (375 patients; 62%). Therefore, we observed no apparent association between CRISP-3 and MSP.

We further analyzed these data to determine if the combination of CRISP-3 and MSP was associated with recurrence. We defined three groups: group 1, CRISP-3–negative and MSP-positive (230 patients); group 2, positive for both CRISP-3 and MSP or negative for both CRISP-3 and MSP (416 patients); and group 3, CRISP-3–positive and MSP-negative (97 patients). The Kaplan-Meier recurrence-free probability stratified by this grouping is shown in Fig. 3C. On multivariable analysis, the hazard ratio for recurrence among patients in group 2 compared with group 1 was 1.72 (95% CI, 1.18, 2.51); the hazard ratio for recurrence among patients in group 3 compared with group 1 was 2.38 (95% CI, 1.48, 3.82; global test for difference between groups, $P = 0.001$). Among patients with complete CRISP-3 and MSP data, the *c* index of the base model was 0.777. Adding this grouping marginally increased the *c* index to 0.779.

To test the hypothesis that MSP was related to the specimen Gleason score, we analyzed the 747 patients with complete MSP and pathologic data. Among patients positive for MSP, 29 (11%) had Gleason score ≥8, 149 (56%) had Gleason score 7, and 87 (33%) had Gleason score ≤6. The proportions were very similar among patients negative for MSP: 46 (10%) had Gleason score ≥8; 263 (55%) had Gleason score 7; and 173 (36%) had Gleason score ≤6. Therefore, the Gleason score did not differ significantly between patients positive and negative for MSP ($P = 0.6$, χ^2). We did a similar analysis for CRISP-3 and found no significant difference in Gleason score between patients positive and negative for CRISP-3 ($P = 0.3$, χ^2). Specifically, among patients positive for CRISP-3, 17 (12%) had Gleason score ≥8; 81 (58%) had Gleason score 7; and 42 (30%) had Gleason score ≤6, which was similar to the proportions among the patients negative for CRISP-3, where 62 (10%) had Gleason score ≥8; 342 (54%) had Gleason score 7; and 231 (36%) had Gleason score ≤6.

Discussion

Accurate predictive models for prostate cancer recurrence after RP are essential for patients counseling and for the rational application of adjuvant therapy. User-friendly nomograms have been developed to predict outcome in prostate cancer patients and in other malignancies, and the software is freely available to the public (32). Recently, an updated postoperative nomogram for predicting the 10-year probability of prostate cancer recurrence after RP was published, but the only biomarker included is the preoperative serum PSA level (7). Recent advances in molecular biology techniques have facilitated the investigation of pathogenesis in malignant diseases. Because the introduction of high-throughput systems, including DNA microarrays, tissue microarrays, and proteomics, a large number of novel biomarkers with diagnostic, prognostic, and therapeutic significance in prostate cancer have been identified (33–38). Addition of tissue markers to nomograms has great potential to improve the prediction of outcome and response to therapy in different malignancies, but thus far, few attempts have been described in the literature.

Reports from two independent groups suggested CRISP-3 to be one of the most up-regulated genes in prostate cancer tissues (16–18), and we recently confirmed this suggestion by IHC, *in situ* hybridization, and Western blot (19). In the same study, however, we found that CRISP-3 was not a useful serum marker for prostate cancer, which can be explained by the high level of circulating CRISP-3 released from different sources, including bone marrow and neutrophil granulocytes (11). Based on our findings of increased expression of CRISP-3 in high-grade tumors with low PSA production (19), we investigated whether tissue expression of CRISP-3 can be used to predict outcome in prostate cancer patients after curative surgery. We were also interested to find whether MSP, which forms high-affinity complexes with CRISP-3 in seminal fluid (12), can serve as a prognostic marker, either alone or in combination with CRISP-3.

A unique feature of this study is the inclusion of a large number of patients with long follow-up. To our knowledge, this is the largest study of its kind, with triplicate samples from almost 1,000 patients treated at a single institution and with complete data available for 80% to 85% of patients. Due to limited prior data on CRISP-3 and MSP, we were unable to prespecify thresholds for defining a tumor as marker positive. We searched across a variety of thresholds to determine whether marker status was associated with outcome, and accordingly, the strength of our findings is tempered by multiple testing. Our data suggesting that both CRISP-3 and MSP can predict outcome in terms of time to BCR after RP replicate previous work suggesting that higher tissue expression of CRISP-3 is a negative prognostic factor in prostate cancer patients.

Besides PSA and prostate acid phosphatase, MSP is one of the three predominant proteins secreted from the benign prostatic epithelium (21). Prior studies on MSP as a biomarker in prostate cancer have produced differing results. Nam et al. (39) studied 1,212 men and found that patients with low PSP94 (MSP) levels in serum had a higher probability for having prostate cancer detected at biopsy. These authors also suggested that those cancers that maintain MSP expression tend to be well differentiated and less aggressive, as already shown in an earlier study from our own group (40). In a more recent study comprising 185 patients, the same group showed that PSPBP, a serum protein that binds MSP, is negatively associated with recurrence after RP, and that both PSPBP and the ratio of bound/free MSP and PSPBP were independent predictors of BCR after adjusting for PSA, Gleason score, and surgical margin status (41). Free and total serum MSP were not found to be significant predictors of recurrence in this study. In contrast to the results with serum MSP, Girvan et al. (29) reported that increased intratumoral expression of the MSP predicts higher risk of prostate cancer recurrence and progression after RP. Our results are in sharp contrast with their findings because we found a high expression of MSP in tumor cells to be associated with a longer time to BCR. The studies are not quite

comparable; as we used different anti-MSP IgGs, the number of tumors examined was only 59 in the study by Girvan et al., compared with 947 in our series, and finally, the methods of scoring the immunostaining and applying statistics were different. Interestingly, our results support a series of experimental studies indicating beneficial effects of MSP, in which MSP and PKC3145, a synthetic peptide of 15 amino acids derived from MSP, were shown to reduce experimental skeletal metastases, prostate tumor growth, and malignancy-associated hypercalcemia in a xenograft model (42,43). Proposed mechanisms of action include the inhibition of matrix metalloproteinase-9 secretion, interaction with the cell surface receptors CD44 and laminin, and inhibition of vascular endothelial growth factor signaling in endothelial cells (44–47). Recently, Beke et al. (48) published intriguing new data suggesting a hitherto unexplored link between MSP and the putative oncogene EZH2, showing that the MSP gene is silenced by EZH2 in advanced prostate cancer cells. Taken together, these results suggest that an association between decreased expression of MSP and prostate cancer is of importance.

The biological importance of the association of CRISP-3 with prostate cancer is unclear because the function of this protein in man remains to be established. CRISP-3 is highly expressed in neutrophil granulocytes, and a role in innate immune defense has been hypothesized (11,13). This hypothesis is supported by its abundant expression in neutrophils and in exocrine glands and by sequence similarities with the so-called pathogenesis-related proteins, which are involved in plant antimicrobial defense. An up-regulation of CRISP-3 expression in tumor cells is interesting in terms of a possible relationship between inflammation and cancer, but this is pure speculation until exploratory studies have been undertaken.

CRISP-1 and CRISP-3 expression in mice is regulated by androgens (49,50). There are, however, structural differences between human and murine CRISP proteins (10), so we cannot assume that CRISP-3 expression in the human prostate is also under the control of androgens. We recently investigated the serum concentration of CRISP-3 before and after orchiectomy in patients with advanced prostate cancer (19). A subset of the patients showed a decreased serum level of CRISP-3 after castration, indicating that the expression of CRISP-3 may be related to androgen receptor function. However, the reduction in the level of CRISP-3 was modest compared with the reduction in the level of serum PSA, a protein whose expression is known to be androgen regulated. The regulation of CRISP-3 expression in the human prostate gland is therefore still unknown.

Expression levels of CRISP-3 and MSP in the benign prostate gland differ markedly. MSP is one of the most abundant proteins secreted from the prostatic epithelium (21), whereas CRISP-3 is expressed at a very low level in the benign epithelium (12). A number of tissue microarray cores in this study contained benign epithelial cells with the previously described pattern of very strong immunostaining for MSP and weak or absent CRISP-3 immunoreactivity (Fig. 1). Therefore, we were also interested in any possible relationship between the expression of CRISP-3 and MSP in individual tumor specimens. We did not, however, find any statistically significant correlation between the expression levels of these proteins based on a univariate association with biochemical failure as an end point, although it was much more common to find an inverse relation between CRISP-3 and MSP than finding both at a high or low expression level. The most favorable finding in terms of outcome was the low expression of CRISP-3 and the high expression of MSP (Fig. 3C). Furthermore, we did not find any statistically significant correlation between Gleason score and CRISP-3 or MSP.

We have previously shown that MSP forms high-affinity complexes with CRISP-3 in seminal fluid (12). In human plasma, however, CRISP-3 is known to form complexes with A1BG (31). A1BG belongs to the immunoglobulin superfamily, and no structural similarity has been found between A1BG and MSP. As noted above, serum MSP has been shown to form a complex with PSPBP (41). Interestingly, the NH_2-terminal part of PSPBP (residues 4–170)

has a sequence similarity with a corresponding part of CRISP-3 (residues 17–183), indicating that this part of the two proteins is responsible for binding to MSP. Based on immunostaining patterns for CRISP-3 and MSP shown in the present study, it would be interesting to investigate whether a complex formation between CRISP-3 and MSP may occur locally in prostatic intraepithelial neoplasia and in a subset of prostate cancers.

Interpretation of IHC is hampered by the lack of a reliable system for quantitation of the signal intensity with enzyme-based methods. To minimize artifactual differences in staining intensity, we used an automated immunostaining machine, and all slides were processed in the same batch under identical conditions after careful optimization of staining protocols. Prostate specimens were tested in triplicate, and cases with fewer than two observations were considered not valid. Although we standardized our methods as much as possible, we realize that there is an urgent need for improvements in signal quantification and computerized image analysis. Although there are no commercially available systems that fulfill all these requirements, promising techniques have been described. PSA, human kallikrein-2, and α-1-antichymotrypsin have been quantified in prostate cancer specimens using time-resolved fluorescence of lanthanide chelates conjugated to primary antibodies or streptavidin (51,52). More recently, several investigators have described nanoparticle reagents under development, including quantum dot conjugates (53).

In the present study, we show that prostate cancer specimens with a relatively high expression of CRISP-3 and a low expression of MSP are associated with a shorter time to BCR. The functional implication of this finding needs to be further explored. As mentioned above, CRISP-3 has been suggested to have a role in innate immune defense (15). Therefore, it would be interesting to further explore a possible role of CRISP-3 in relation to inflammation and tumor development. A vast majority of cells in HGPIN lesions express both CRISP-3 and MSP, and this may indicate that a change in the balance between these proteins precedes tumor development. Expression of both proteins provides the opportunity for CRISP-3 and MSP to form high-affinity complexes, but the function of these complexes is still unknown. As we found that high tumor expression of CRISP-3 in combination with low expression of MSP was related to unfavorable outcome, one can hypothesize that uncomplexed CRISP-3 in tissue may be related to tumor progression.

Conclusions

We report evidence that the complexing proteins CRISP-3 and MSP are independent predictors of recurrence after RP for localized prostate cancer. However, addition of the markers to the established predictors of outcome does not improve the performance of existing predictive models, suggesting that the markers are unlikely to be important as prognostic factors in the clinic. Nonetheless, given the biological association between the markers and outcome, an evaluation of these findings in an independent data set is warranted. If such studies are confirmatory, further research should aim to elucidate the function of CRISP-3 and MSP expression in prostate cancer cells.

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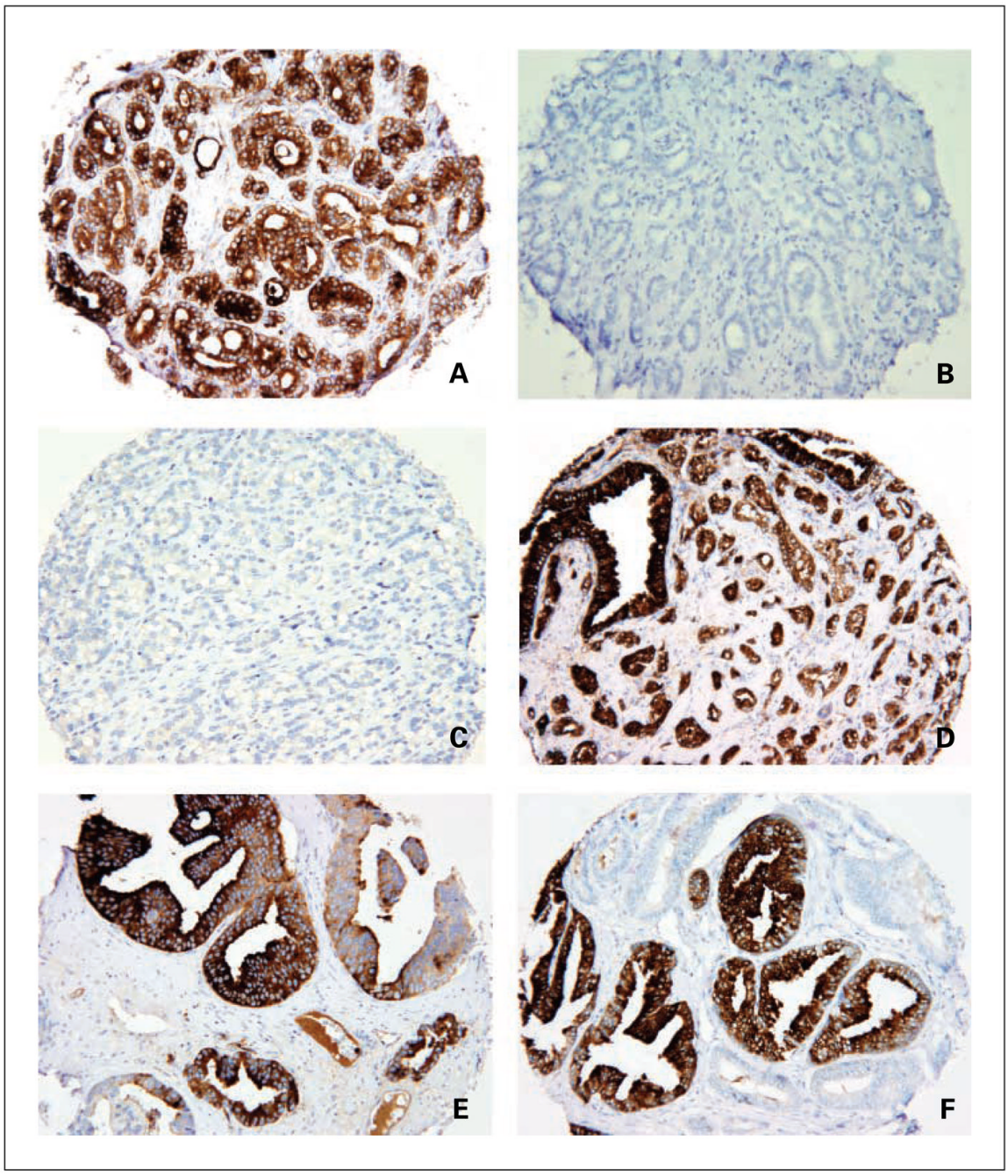

Fig. 1.

Immunohistochemical detection of CRISP-3 and MSP in tissue microarray cores of primary tumors and HGPIN from RP specimens. *A*, a Gleason grade 4 tumor with strong immunostaining for CRISP-3 in most prostate cancer cells. *B*, an adjacent section of the same tumor as in (*A*), with virtually no detectable MSP immunoreactivity. *C*, another example of a Gleason grade 4 tumor, with almost complete lack of immunoreactivity for CRISP-3. *D*, Gleason grade 4 tumor cells and benign epithelium with strong expression of MSP. *E*, an HGPIN lesion, showing CRISP-3– immunoreactive cells. *F*, MSP immunoreactive cells in HGPIN. Magnification, ×200.

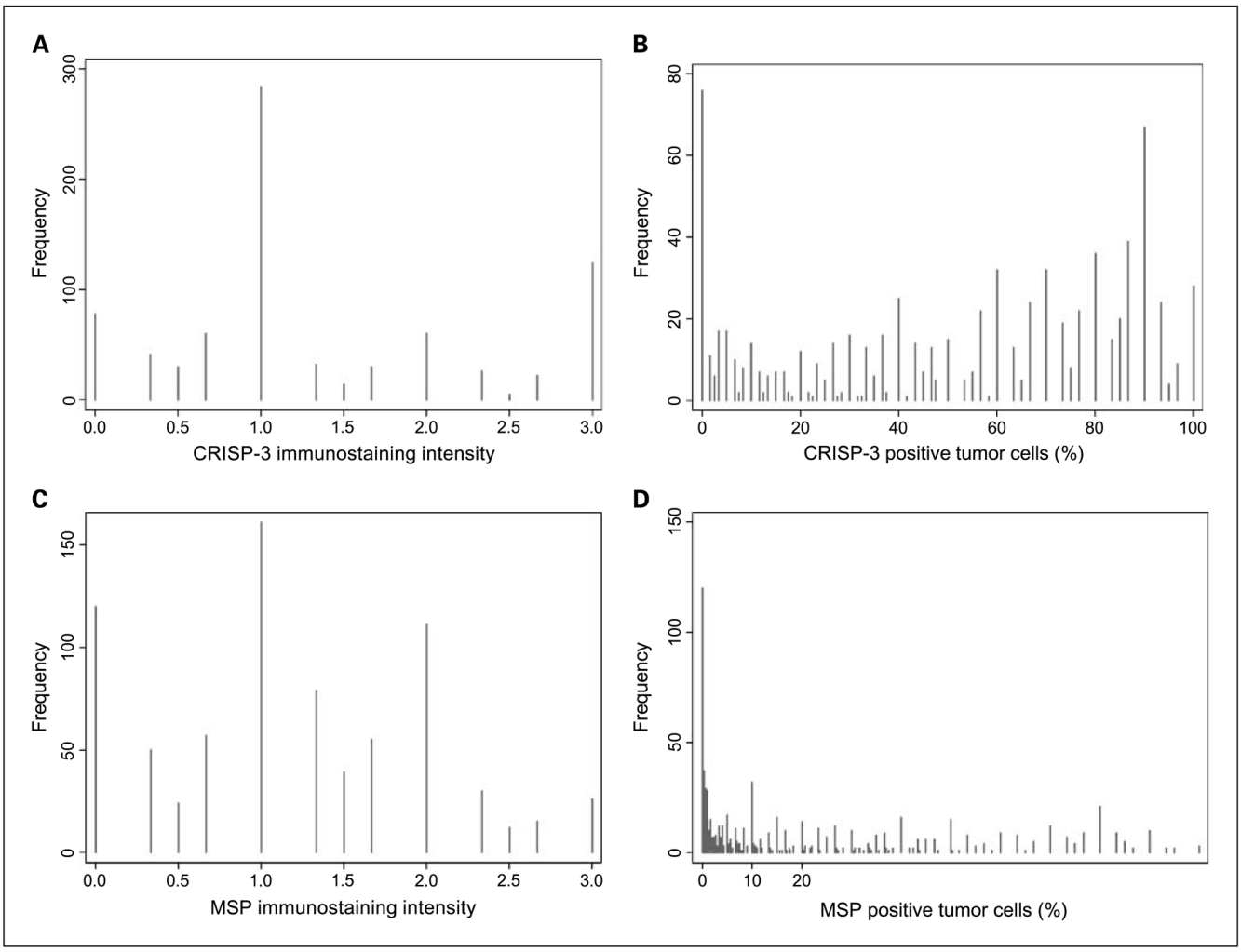

Fig. 2.

Results from semiquantitative scoring of tissue microarrays. *A*, distribution of CRISP-3 immunostaining intensity. *B*, percentage of tumor cells positive for CRISP-3. *C*, MSP immunostaining intensity. *D*, Percentage of MSP-positive tumor cells.

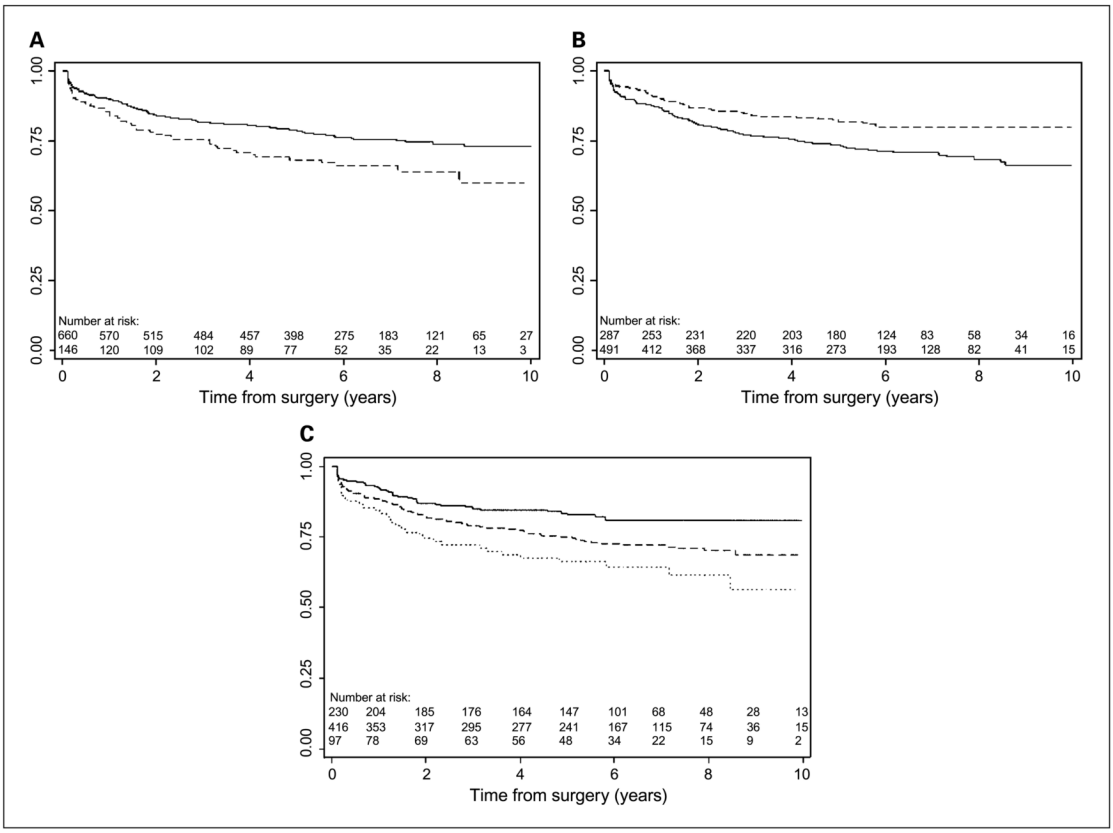

Fig. 3.

Kaplan-Meier recurrence-free probability. *A*, patients stratified by CRISP-3 positivity, defined as ≥80% of tumor cells positive and immunostaining intensity ≥1.5. Solid line, CRISP-3 negative. Dashed line, CRISP-3 positive. *B*, patients stratified by MSP positivity, defined as ≥20% of tumor cells positive and immunostaining intensity ≥1.0. Solid line, MSP negative. Dashed line, MSP positive. *C*, patients stratified by the combination of CRISP-3 and MSP. Solid line, group 1, CRISP-3 negative, and MSP positive. Dashed line, group 2, both CRISP-3 and MSP negative or both CRISP-3 and MSP positive. Dotted line, group 3, CRISP-3 positive and MSP negative.

Table 1

Clinical and pathologic characteristics of the 945 patients analyzed

Characteristic	Median (IQR) or *n* (%)
Age at surgery (y)	62 (56, 66)
Preoperative PSA (ng/mL)	7.8 (5.6, 12.7)
Clinical stage	
T1	475 (50)
T2	250 (26)
T3	220 (23)
Biopsy Gleason score	
≤6	610 (64)
7	273 (29)
≥8	62 (7)
Pathology Gleason score*	
≤6	312 (33)
7	472 (50)
≥8	84 (9)
Extracapsular extension* (%)	258 (30)
Seminal vesicle invasion* (%)	78 (9)
Positive surgical margins* (%)	299 (34)
Lymph node involvement* (%)	27 (3)

Abbreviation: IQR, interquartile range.

*Pathologic data were available for only 868 patients.

Table 2

Univariate Cox proportional hazards regression to evaluate the association between CRISP-3 and MSP and BCR after prostatectomy

Cut-point*	CRISP-3			MSP		
	Number positive (%)	*P*	Hazard ratio (95% CI)	Number positive (%)	*P*	Hazard ratio (95% CI)
% of tumor cells positive	*n* = 806, with recurrence = 198			*n* = 778, with recurrence = 190		
10	659 (82)	0.8	0.95 (0.67, 1.34)	386 (50)	0.054	0.75 (0.57, 1.00)
20	613 (76)	0.6	1.09 (0.78, 1.53)	289 (37)	0.009	0.66 (0.48, 0.90)
30	567 (70)	0.3	1.19 (0.87, 1.64)	229 (29)	0.017	0.66 (0.47, 0.93)
40	512 (64)	0.13	1.26 (0.94, 1.70)	183 (24)	0.044	0.68 (0.47, 0.99)
50	447 (55)	0.02	1.39 (1.04, 1.85)	142 (18)	0.2	0.77 (0.52, 1.14)
60	397 (49)	0.08	1.28 (0.97, 1.70)	109 (14)	0.3	0.78 (0.50, 1.21)
70	323 (40)	0.11	1.26 (0.95, 1.67)	86 (11)	0.4	0.83 (0.51, 1.34)
80	242 (30)	0.048	1.34 (1.01, 1.80)	54 (7)	0.2	0.65 (0.33, 1.28)
90	132 (16)	0.09	1.36 (0.96, 1.93)	17 (2)	—†	—†
Immunostaining intensity	*n* = 806, with recurrence = 198			*n* = 779, with recurrence = 191		
0.5	687 (85)	0.8	1.05 (0.71, 1.55)	609 (78)	0.3	0.83 (0.60, 1.15)
1.0	597 (74)	0.9	1.00 (0.73, 1.38)	528 (68)	0.3	0.86 (0.64, 1.15)
1.5	281 (35)	0.5	1.11 (0.83, 1.48)	288 (37)	0.3	0.85 (0.63, 1.15)
2.0	237 (29)	0.8	1.04 (0.77, 1.41)	194 (25)	0.6	0.90 (0.65, 1.27)
2.5	151 (19)	0.18	1.26 (0.90, 1.76)	53 (7)	0.8	1.08 (0.63, 1.86)
Combination	*n* = 806, with recurrence = 198			*n* = 778, with recurrence = 190		
≥20% positive and intensity ≥1.0	—	—	—	287 (37)	0.004	0.63 (0.46, 0.86)
≥30% positive and intensity ≥1.0	—	—	—	229 (29)	0.017	0.66 (0.47, 0.93)
≥70% positive and intensity ≥1.5	190 (24)	0.030	1.41 (1.04, 1.92)	—	—	—
≥80% positive and intensity ≥1.5	146 (18)	0.010	1.53 (1.11, 2.12)	—	—	—

*Patients are positive if marker is greater than or equal to the cut-point.

†The model did not converge because none of the 17 MSP-positive patients had a recurrence.